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Original article

Modulation of reactive oxygen species via ERK and STAT3 dependent signalling are involved in the response of *meso*thelioma cells to exemestane

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ABSTRACT

Pleural *meso*thelioma is a deadly form of cancer. The prognosis is extremely poor due to the limited treatment modalities. Uptake of asbestos fibres, the leading cause of *meso*thelioma, lead to the accumulation of reactive-oxygen-species (ROS). Interestingly, increasing ROS production by using ROS-generating drugs may offer a strategy to selectively trigger cell death. Exemestane, an aromatase inhibitor, has previously shown anti-tumor properties in *meso*thelioma preclinical models suggesting a role of G protein-coupled receptor 30 (GPR30) in the drug response. As exemestane, in addition to blocking estrogen biosynthesis, generates ROS that are able to arrest the growth of breast cancer, we explored the role of ROS, antioxidant defense system, and ROS-induced signalling pathways in *meso*thelioma cells during exemestane response. Here we report that exemestane treatment reduced cell proliferation with an increase in ROS production and reduction of cyclic adenosine monophosphate (cAMP) levels in MSTO-H211, Ist-Mes1, Ist-Mes2 and MPP89 exemestane-sensitive *meso*thelioma cell lines, but not in NCI-H2452 exemestane-insensitive *meso*thelioma cells. Exemestane induced a significant antioxidant response in NCI-H2452 cells, as highlighted by an increase in γ-glutamylcysteine levels, catalase (Cat), superoxide-dismutase and (SOD) and glutathione-peroxidase (GSH-Px) activity and nuclear factor E2-related factor 2 (Nrf2) activation, responsible for drug insensitivity. Conversely, exemestane elevated ROS levels along with increased ERK phosphorylation and a reduction of p-STA3 in exemestane-sensitive *meso*thelioma cells. ROS generation was the crucial event of exemestane action because ROS inhibitor N-acetyl-L-cysteine (NAC) abrogated p-ERK and p-STAT3 modulation and cellular death. Exemestane also modulates ERK and STAT3 signalling via GPR30. Results indicate an essential role of ROS in the antiproliferative action of exemestane in *meso*thelioma cells. It is likely that the additional oxidative insults induced by exemestane results in the lethal effects of *meso*thelioma cells by increasing ROS production. As such, manipulating ROS levels with exemestane seems to be a feasible strategy to selectively kill *meso*thelioma cells with less toxicity to normal cells by regulating ERK and STAT3 activity.

1. Introduction

Malignant *meso*thelioma (MM) is a lethal tumor arising from the *meso*thelium of the serous cavities. MM occurrence is closely associated with exposure to asbestos. Free radicals and reactive oxygen species (ROS), key mediators of the tissue damage caused by asbestos, are associated with abnormal cancer cell growth due to disruption of redox homeostasis with an increase in ROS production and a decrease in the activity of the ROS scavenging system [1,2]. Because oxidative stress is involved in the initiation and progression of cancer [3] it is considered an adverse event. On the other hand, excessive oxidative stress can cause cell death [4]. Following treatment with various agents, a significant increase in the intracellular ROS production and downstream acidification provides an environment conducive for apoptotic cell death in tumor cells. Therefore, increasing the cellular levels of ROS by using ROS-generating drugs may offer a strategy to selectively kill cancer cells [5]. Consistently, cancer cells inherently endowed with oxidative stress are more susceptible than normal cells to oxidative insults delivered by exogenous agents that generate ROS. Thus, by manipulating the levels of ROS cancer cells can be selectively killed without causing significant toxicity to the normal tissue [6]. The anticancer effect of various chemotherapeutic agents currently used (e.g.,

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paclitaxel, cisplatin, arsenic trioxide, etoposide, doxorubicin) is mediated, at least in part, by an increase in the cellular levels of ROS [7,8].

ROS, including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) are constantly generated under normal conditions as a result of aerobic metabolism [9]. Increased ROS production due to their high levels of chemical reactivity, cause deterioration of the structure and functions of DNA, proteins, carbohydrates, and lipids, which overall results in increased mutation rates, promotion of oncogenic transformation, and contribution to the development of cancer and metastases [10]. Endogenous non enzymatic antioxidants such as reduced glutathione (GSH) and its precursor γ-glutamylcysteine (γ-GC) normally counteract the damaging effects of intracellular ROS either as part of the repairing armamentarium of the oxidative damage or by directly scavenging oxygen radicals. Detoxification of $O_2^{\bullet -}$ and H_2O_2, catalyzed by intracellular superoxide dismutase (SOD), catalase (Cat) and glutathione peroxidase (GSH-Px) enzyme activity represent a major line of enzymatic defense. Disruption of the pro-oxidant/antioxidant balance in favor of the former one, leads to potential damage called oxidative stress, which is involved in the development of cancer [11].

MM is nearly always, except in rare less-advanced cases, with a median survival of 12.6 months [12]. The prognosis of MM patients remains poor despite clinical treatments including surgery, radiotherapy and chemotherapy as reported [13]. Thus, to improve the clinical outcome of in the pharmacological treatment of this refractory tumor, drugs directed against novel tumor-specific cellular targets and/or characterized by more specific mechanisms of action are needed. We have previously reported that exemestane (6-methyleneandrosta-1,4-diene-3,17-dione), an aromatase inhibitor (AI), elicited apoptosis in MM cells and in mice MM xenografts, and that the association of exemestane/pemetrexed was more effective than pemetrexed/cisplatin [14,15]. Encouraging results have led us to a better understanding of the mechanism of action of exemestane in MM cells. Oxidative stress is known to be involved in the exemestane mechanism of action in breast cancer cell lines [16]. In turn, ROS are involved in pAKT and cyclic adenosine monophosphate (cAMP) modulation [17,18]. AKT activation and cAMP modulation have been described in the action of exemestane in MM cells [14,15]. Thus, in this study we investigated the involvement of ROS and the related signalling pathways in response to exemestane and show that exemestane acts through ROS production. Antioxidant enzymes and the glutathione biosynthetic pathway as well as nuclear factor E2-related factor 2 (Nrf2), which controls the expression of antioxidant proteins [19], were implicated in the exemestane sensitivity of *meso*thelioma cells. Several reports have highlighted an effect of ROS on the signal transducer and activator of transcription 3 (STAT3) activity [20–23]. Of note, STAT3 has been reported to be a target of estrogen signalling [24–26]. STAT proteins are activated by phosphorylation at a specific tyrosine residue at the carboxy-terminus, required for dimerization, nuclear translocation, DNA binding and transcriptional activity. STAT3 is phosphorylated and activated in response to estrogen in a process involving multiple intracellular signal pathways. Mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) [26] may negatively regulate STAT3 activity by decreasing its tyrosine phosphorylation [27]. Moreover, increased intracellular ROS production leads to the activation of ERK proteins. Nevertheless, the mechanisms by which ROS can activate these kinases are unclear [28]. Given the involvement of ROS in response to exemestane, in this study we evaluated the action of ROS on ERK activity. Simultaneously, we tested the effect of 17-β-estradiol (E2) on ERK phosphorylation.

Furthermore, we investigated the role of GPR30 on the ERK activation since estrogen-mediated adenylyl cyclase stimulation (that produces cAMP) occurs independently from known estrogen receptors but instead requires G protein-coupled receptor 30 (GPR30) also known as G protein-coupled estrogen receptor 1 (GPER) [29], suggested as being involved in the response of MM cells to exemestane [15,30].

2. Material and methods

2.1. Materials

Exemestane was from Sequoia Research (Pangbourne, UK). 2',7'-dichlorofluorescin diacetate (DCFH-DA), buthionine sulfoximine (BSO) and N-acetyl-L-cysteine (NAC) were purchased from Signa-Aldrich (Milan, Italy). cAMP ELISA Kit was from R&D Systems (Minneapolis, MN, USA). NE-PER nuclear and cytoplasmic extraction kit was from Pierce-Thermo, (Northumberland, UK). Complete, protease inhibitor cocktail and PhosSTOP™, inhibitor for phosphatase were provided from Sigma-Aldrich (Milan, Italy). $p21^{WAF1/CIP1}$ (p21), $p27^{Kip1}$(p27), cleaved PARP, caspase3, Nrf2, STAT and p-STAT (phospho-STAT3 (Tyr705)) antibodies were obtained from Cell Signalling Technology (Danvers, USA), Gamma and α-tubulin were acquired from Sigma-Aldrich (Milan, Italy). Goat anti-mouse and rabbit IgG horseradish peroxidise conjugate secondary antibodies were purchased from Bio-Rad Laboratories. Enhanced ChemiLuminescence (ECL) was from Amersham Biosciences (Piscataway, USA).

2.2. Cell lines and cell culture

Human *meso*thelioma cell lines MSTO-211H and NCI-H2452 were obtained from the American Type Culture Collection (ATCC) while Ist-Mes1, Ist-Mes2, and MPP89 were obtained from the Genova Institute Culture Collection. Cell lines were periodically tested for mycoplasma contamination by MycoFluor™ Mycoplasma Detection Kit (Thermo Fischer) and cultured as described previously [31]. Cell morphology was monitored routinely and compared to cell morphology images, growth curve analysis were evaluated periodically. Before treatment upon exemestane, all cell lines were gradually conditioned in DMEM/F12 + Glutamax (Invitrogen) supplemented with 10% FBS and antibiotics.

2.3. Cell treatments

The drug treatment was assessed in a monolayer culture condition by plating NCI-H2452, Ist-Mes1, Ist-Mes2, MSTO-211H, and MPP89 cell lines in T25 flask. After 24 h, vehicle dimethyl sulfoxide (DMSO), 35 μM exemestane or 100 μM H_2O_2 or 100 μM BSO or 5 mM NAC were added for the time indicated in the experiments. We chose the pharmacological concentration of 35 μM exemestane. 100 μM H_2O_2 or 100 μM BSO or 5 mM NAC had no effect on the cellular proliferation. The expansion of culture cell proliferation was quantified by manual cell counting. Experiments were repeated in triplicate and results were averaged. Cells were also treated with 10 nM E2 or 10 nM E2 and 100 nM G15. G15 was added 10 min before E2.

2.4. Detection of ROS production

The DCFH-DA fluorescent probe was employed to detect ROS. The cell-permeable DCFH-DA passively diffuses into cells and is retained in the intracellular level after cleavage by intracellular esterases. Oxidation of DCFH-DA by ROS converts the molecule to 2', 7' dichlorodihydrofluorescein (DCF), which is highly fluorescent [31]. MM cells were plated in 96 micro-well plates at the density 10,000 cells in 200 μl of DMEM per well and incubated for 24 h at 37 °C and 5% CO_2. The cells were washed with 150 μl phosphate buffered saline (PBS) and incubated for 30 min with 100 μl PBS containing 5 μM DCFH-DA. Cells were then washed again with 150 μl PBS to remove the exceeding probe prior of treatment in 200 μl of DMEM with 35 μM exemestane, 5 mM NAC or a combination of the drug with the antioxidant agent for various time periods. Fluorescence was measured after 30 min, 1 h and 4 h on the automated 96-well plate reader (Fluoroskan Ascent FLTM, Labsystems) using an excitation and an emission wavelength of 485 nm, and 530 nm, respectively. ROS levels were calculated as a percent